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Bacterial Diversity and Function of Aerobic Granules Engineered in a Sequencing Batch Reactor for Phenol Degradation

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Aerobic granules are self-immobilized aggregates of microorganisms and represent a relatively new form of cell immobilization developed for biological wastewater treatment. In this study, both culture-based and culture-independent techniques were used to investigate the bacterial diversity and function in aerobic phenol-degrading granules cultivated in a sequencing batch reactor. Denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S rRNA genes demonstrated a major shift in the microbial community as the seed sludge developed into granules. Culture isolation and DGGE assays confirmed the dominance of β-*Proteobacteria* and high-G+C gram-positive bacteria in the phenol-degrading aerobic granules. Of the 10 phenol-degrading bacterial strains isolated from the granules, strains PG-01, PG-02, and PG-08 possessed 16S rRNA gene sequences that matched the partial sequences of dominant bands in the DGGE fingerprint belonging to the aerobic granules. The numerical dominance of strain PG-01 was confirmed by isolation, DGGE, and in situ hybridization with a strain-specific probe, and key physiological traits possessed by PG-01 that allowed it to outcompete and dominate other microorganisms within the granules were then identified. This strain could be regarded as a functionally dominant strain and may have contributed significantly to phenol degradation in the granules. On the other hand, strain PG-08 had low specific growth rate and low phenol degradation ability but showed a high propensity to autoaggregate. By analyzing the roles played by these two isolates within the aerobic granules, a functional model of the microbial community within the aerobic granules was proposed. This model has important implications for rationalizing the engineering of ecological systems.

Phenol is a major pollutant in industrial wastewater, and its removal is of obvious interest. Biological treatment of phenol is generally preferred to physical or chemical treatment methods because of lower costs and the possibility of complete mineralization. However, conventional biological treatment systems such as the activated sludge process are known to be sensitive to high phenol loading rates and fluctuations in phenol loading due to substrate inhibition from phenol toxicity (44).

These substrate inhibition difficulties can be overcome by strategies such as cell immobilization to protect microbial cells against phenol toxicity (24). Aerobic granulation is a recently reported form of cell immobilization technology that is attracting considerable research attention (27, 38). Aerobic granules are self-immobilized aggregates of microorganisms formed in engineered systems such as sequencing batch reactors (SBRs). Unlike activated sludge flocs, microbial granules have a well-defined appearance and are still visible as separate entities after settling (9). Granulation facilitates the accumulation of high amounts of active biomass and the effective separation of this biomass from the wastewater liquor. Early studies of aerobic granulation involved the use of benign substrates such as glucose and acetate (38, 39). Several recent studies reported the successful cultivation of aerobic granules using toxic phenol as a substrate and examined the effect of phenol loading on granule structure, activity, and metabolism (13, 14).

While the microbial diversities of glucose-fed aerobic granules and phenol-degrading activated sludge have been reasonably well described (39, 43, 44), a gap in our understanding of the microbial communities residing in phenol-degrading aerobic granules still exists. Aerobic granules can be viewed as a special form of biofilm but without carriers for biofilm attachment. Growth environments for biofilm communities are different from those for planktonic communities, and microbial communities in attached biofilms have been shown to be highly distinct from the suspended biomass, even within a single reactor system (4, 8). Recent studies stress the importance of gaining an understanding of the functions of microbial communities, as population diversity alone may not be adequate in maintaining ecosystem stability. Recognizing the diversity and the links within the key functional groups in a given system can lead to better ways to model diversity and function as well as to improve process stability (3, 12, 16).

In this study, culture-independent and culture-dependent methods were used in combination to study the microbial community of phenol-degrading aerobic granules and to isolate, characterize, and identify ecologically relevant microorganisms. One of the isolates demonstrated a strong ability to degrade phenol and maintained a dominant presence within the granule community. A second isolate showed a weak ability to degrade phenol but was exceptional in its ability to autoaggregate. Based on these observations, a functional model of the microbial community in the aerobic granules was proposed. This work is expected to be useful in understanding the ecology and function of aerobic granules and in developing optimal control and management strategies for aerobic granulation systems.

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MATERIALS AND METHODS

Reactor operation. Phenol-degrading aerobic granules were cultivated in an SBR from activated sludge seed, which was first conditioned by incubation with phenol which gradually increased in concentration from 50 to 500 mg liter^{-1}, as

described previously (13). The reactor was operated at a loading rate of 1.5 kg of phenol m^{-3} day^{-1} with an influent phenol concentration of 500 mg $liter^{-1}$ and reached steady state after 2 months of operation. Mature granules had diameters that ranged from 0.4 to 0.6 mm. The granules were sampled during this steady state for both cultivation and ribosomal-based experiments.

Analytical methods. Measurements of phenol concentrations, volatile suspended solids (VSS), polysaccharides, and protein in extracellular polymers (ECPs) were performed as described previously (14).

Isolation procedure. The culture medium used for isolation and growth on phenol was prepared by using modified MP medium containing (per liter) 1.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgCl_2 \cdot 6H_2O$, 0.1 g of NaCl, 0.02 g of $FeCl_3 \cdot 6H_2O$, 0.01 g of $CaCl_2$, and phosphate buffer (1.35 g of KH_2PO_4 and 1.65 g of K_2HPO_4) (43), supplemented with trace elements and vitamins (5). The medium was sterilized by autoclaving for 20 min at 121°C. The phenol solution was filter sterilized and added to the autoclaved medium.

Portions of mature granules were added to 15 ml of MP medium and aseptically mixed in a sterilized beaker in order to detach granules. The supernatant was then serially diluted with medium (10^1- to 10^7-fold dilutions), and 150 μl of each dilution was spread onto agar plates containing MP medium (supplemented with 500 mg of phenol per liter) solidified with 1.2% Bacto agar (Difco, Detroit, Mich.). The plates were inverted and incubated in a 25°C incubator (Sanyo, Osaka, Japan) and monitored over 4 weeks. Visible colonies were observed after 1 week of incubation. Pure cultures of phenol-degrading bacteria were isolated by cycles of replating onto MP-phenol agar plates. Purity was confirmed by examination with both light microscopy (BX-FLA-3 epifluorescence microscope; Olympus, Tokyo, Japan) and scanning electron microscopy (Stereoscan 420; Leica Cambridge Instruments), as described previously (13). Gram staining was performed as described previously (26). Pure cultures were stored in 50 mM KH_2PO_4-K_2HPO_4 buffer (pH 7.2) containing 20% (vol/vol) glycerol at −70°C. Genomic fingerprints of isolates were obtained by repetitive extragenic palindromic sequence PCR (rep-PCR) performed with primer BOX AIR (5′-CTAC GGCAAGGCGACGCTGACG-3′) (42).

Specific growth and phenol degradation rates. Cells used in the kinetics experiment were exponential-phase cultures grown in MP medium with phenol as the sole carbon and energy source. Experiments were performed in triplicate in 500-ml serum bottles. Each bottle contained 100 ml of MP medium with an initial phenol concentration of 250 mg $liter^{-1}$. Individual strains were introduced into the medium, and all reagent bottles were capped immediately and incubated on an orbital shaker (150 rpm) in the dark at 25°C. Phenol concentrations were assayed at regular intervals during incubation. Cell growth was monitored by optical density (OD) measurement with a spectrophotometer (Perkin-Elmer) at a wavelength of 600 nm. The linear relationship between the OD and total cell numbers counted with DAPI (4′,6′-diamidino-2-phenylindole) stain (47) was confirmed with two isolates. In the case of strain PG-01, the cell density (in cells per liter) was determined and equaled $5.63 \times 10^{11} \times$ OD at 600 nm (OD_{600}) ($R^2 = 0.991$).

The specific growth and phenol degradation rates were calculated by performing a linear regression on initial curves of cell growth and phenol disappearance, respectively, against time. Biomass concentrations on a dry weight basis were determined by filtering the cell suspension through a 0.2-μm-pore filter (cellulose acetate membrane filter; Advantec MFS, Inc. Dublin, Calif.) and drying the filter and cells to a constant weight for 24 h at 80°C (31). A linear relationship between the OD and dry weight was observed. The biomass concentration (g [dry weight] $liter^{-1}$) was calculated from OD measurements and equaled $0.574 \times OD_{600}$ ($R^2 = 0.984$) for strain PG-01.

Autoaggregation activity. To determine autoaggregation activities of isolates grown on phenol as the sole carbon source or yeast extract-peptone-glucose (YEPG) medium, isolates were incubated for 1 day by reciprocal shaking at 60 rpm and then allowed to settle for 1 min (44). Autoaggregation activity was judged based on whether flocculated biomass could be observed.

DNA extraction. Genomic DNA of isolates cultivated in MP-phenol or YEPG liquid medium was extracted by using the bead-beating method with a Mini BeadBeater (Biospec Products) as described previously (39). DNA was extracted from phenol-conditioned activated sludge seed and granule samples by using the same method but with a modified procedure. After bead beating, the sample suspensions were supplemented with 0.05 g of lysozyme (20,000 U/mg; Amersham, Uppsala, Sweden) and incubated at 37°C for 20 min followed by phenol extraction. The extracted DNA was stored at −20°C. Replicate DNA samples were extracted from granules, activated sludge, and isolates.

16S rRNA gene sequencing and phylogenetic analyses. A whole-cell direct lysis PCR amplification method was used to amplify the 16S rRNA gene of isolates as described previously (26). The nearly full-length 16S rRNA gene was amplified by PCR with forward primer Eubac27F and reverse primer Universal 1492R1 (18). The PCR products were purified with a PCR purification kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions and stored at −20°C before sequencing.

TABLE 1. Homologous 16S rRNA sequences of probe Pand822, its target, and its closest relatives

Organism or sequence	Probe or target sequence[a]
Probe (3′–5′)	3′-AGGTGATCGACGACCCCTAA-5′
Target sequence (5′–3′)	5′-UCCACUAGCUGCUGGGGAUU-3′
Isolate PG-01	5′-UCCACUAGCUGCUGGGGAUU-3′
Pandoraea apista strain LMG 16407 (AF139173)	5′---A-----U--U---------3′
Pandoraea sp. AU1775 (AY043377)	5′---A-----U--U---------3′
Pandoraea norimbergensis (AY268174)	5′---A-----U--U---------3′
Pandoraea sp. G8107 (AF247695)	5′---A-----U--U---------3′
Pandoraea pnomenusa (AF139174)	5′---A-----U--U---------3′
Pandoraea pulmonicola (AF139175)	5′---A-----U--U---------3′
Pandoraea sputorum (AF139176)	5′---A-----U--U---------3′
Burkholderia cepacia strain LMG 14294 (AF097553)	5′---A-----U--U---------3′
Burkholderia pseudomallei (Ara + biotype) (AF093051)	5′---A-----U--U---------3′
Burkholderia sp. MN 182.2 (AJ313026)	5′---A-----U--U---------3′
Burkholderia phenazinium (U96936)	5′---A---G-U--UC---UC---3′
Burkholderia sacchari (AF263278)	5′---A-----U--UC---CC---3′
Burkholderia stabilis (AF148554)	5′---A-----U--U---------3′
Burkholderia vietnamiensis (U96929)	5′---A-----U--U---------3′
Uncultured eubacterium WD258 (AJ292640)	5′---A-----U--U---------3′
Pseudomonas cepacia (L28675)	5′---A-----U--U---------3′
Pseudomonas caryophylli (X67039)	5′---A-----U--U---------3′

[a] Only mismatching nucleotides are indicated. Matching nucleotides are indicated with a dash.

The 16S rRNA gene sequence of the isolates was determined by using an ABI model 310A DNA sequencer (Applied Biosystems/Perkin-Elmer) and an ABI PRISM BigDye Terminator Ready Reaction cycle sequencing kit (version 3.0; Applied Biosystems/Perkin-Elmer). Phylogenetic analysis of these sequences was performed as described previously (39).

PCR and denaturing gradient gel electrophoresis (DGGE). PCR primers P2 and P3 with 40 bases of a GC clamp were used to amplify the V3 region of the bacterial 16S rRNA gene corresponding to positions 341 and 534 in the *Escherichia coli* sequence (28). Amplification was performed with a Mastercycler gradient (Eppendorf AG, Hamburg, Germany) using a 50-μl (total volume) mixture containing 1.25 U of *Taq* polymerase (Promega), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 μM deoxynucleoide triphosphate (dNTP), 25 pmol of each primer, and 1 μl of DNA solution (20 ng/μl) extracted from activated sludge, mature granules, or isolated strains. Touchdown PCR was employed (28), and the amplicons obtained were confirmed by electrophoresis through 2% agarose gel in 1× TAE buffer (40 mM Tris base, 20 mM sodium acetate, 1 mM Na_2-EDTA, pH 8.0) stained with ethidium bromide. To detect *Archaea*, *Archaea*-specific primers PRA46f and PREA1100r were used to amplify the 16S rRNA gene of *Archaea* under conditions described previously (32).

The PCR-amplified DNA fragments were separated by DGGE with a DCode universal mutation detection system (Bio-Rad Laboratories) as described previously (28) but with several modifications. The 25-ml 30 to 70% urea-formamide denaturant gradient gel (10% [wt/vol] acrylamide solution [40% acrylamide-bisacrylamide, 37.5:1 stock solution; Bio-Rad Laboratories] in TAE buffer) was covered by a 4-ml acrylamide stacking gel (10%) without denaturant. Twenty microliters of PCR amplicon from DNA of pure cultures or 40 μl of PCR amplicon from DNA extracted from aerobic granules or activated sludge was loaded into each well with 20 μl of loading dye. The gel was placed in TAE buffer and run at 40 V at 60°C for 30 min and then at 85 V at 60°C for 14 h. After electrophoresis, the gel was stained with ethidium bromide for 30 min and viewed and photographed with an EDAS 290 gel imaging system (Kodak). Selected DNA bands were excised, and rep-PCR was performed as described previously (43) to obtain partial sequence information. The partial sequences were assembled by using BioEdit software and analyzed with BLAST and other algorithms as described previously (39).

Oligonucleotide probes. An oligonucleotide probe designed to target the 16S rRNA gene of strain PG-01 was generated after comparison of the aligned sequence of PG-01 with the sequences of other bacteria. Probe specificity (Table

1) was verified by using the CHECK_PROBE program (22). The probe exhibited at least three nucleotide mismatches with other currently available 16S rRNA reference sequences. The probe was named S-St-Pand-0822-a-A-20 (Pand822) (5′-AATCCCCAGCAGCTAGTGGA-3′) according to the nomenclature of Alm et al. (1). EUB338 (5′-GCTGCCTCCCGTAGGAGT-3′) and ARCH915 (5′-GTGCTCCCCCGCCAATTCCT-3′), which target highly conserved regions of most bacterial 16S rRNA and most archaeal 16S rRNA, respectively (2), were also used. All probes were made and labeled by Genset (Paris, France). Probe Pand822 was labeled at the 5′ end with the indocarbocyanine dye Cy5, while probes EUB338 and ARCH915 were labeled with fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC), respectively.

Fixation and cryosectioning of aerobic granules. Intact aerobic granules and pure culture cells harvested at the exponential phase were washed in 1× phosphate-buffered saline and then fixed by using freshly prepared paraformaldehyde (Sigma) solution (4% [wt/vol]) at 4°C for 6 and 3 h, respectively, followed by storage in 1× phosphate-buffered saline-ethanol (1:1) at −20°C (2).

The fixed granules were embedded for cryosectioning in optimum cutting temperature compound (Miles, Elkhart, Ind.) by immersion. Embedded samples were frozen overnight at −20°C, after which 20-μm sections were cut on a cryomicrotome (CM3050S; Leica) and mounted onto a gelatin-coated (0.1% gelatin and 0.01% chromium potassium sulfate) microscopic slide. The optimum cutting temperature compound was removed by immersion in Milli-Q water. Five microliters of fixed cells was directly immobilized on a gelatin-coated microscopic slide and air dried. The cells and granule sections were then dehydrated by sequential immersion of the slides for 3 min in 50, 80, and 98% ethanol and air dried.

Fluorescence in situ hybridization (FISH) and optimization of hybridization conditions. Whole-cell hybridization with fluorescently labeled oligonucleotides was performed as described previously by Manz et al. (23). Hybridizations for EUB338 and ARCH519 probes were performed with hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 7.4], 0.01% sodium dodecyl sulfate) containing 5 ng of probe μl^{-1} for 2.5 h at 46°C in an isotonically equilibrated humidity chamber. Hybridization stringency was adjusted by adding formamide (Promega Corporation, Madison, Wis.) to the hybridization buffer (10% for EUB338 and 30% for ARCH519) (2). This step was followed by a 20-min washing step at 48°C in wash buffer before a final wash in Milli-Q water. To achieve the same stringency during washing as that during hybridization, NaCl concentrations in the washing buffer and formamide concentration in the hybridization buffer were adjusted according to the formula of Lathe (19). Wash buffer was prepared in a fresh 50-ml polypropylene tube containing 1 ml of 1 M Tris-HCl (final concentration, 20 mM [pH 7.2]), 50 μl of 10% sodium dodecyl sulfate (final concentration, 0.01%), and 5 M NaCl, with Milli-Q water up to 50 ml.

Hybridization conditions were optimized for probe Pand822 as follows. First, hybridization temperatures were increased from 37 to 65°C in 2 or 5°C steps without formamide in the hybridization buffer to determine a suitable range of hybridization temperatures. The washing temperature was set at 2°C higher than the hybridization temperature. Based on the results, the hybridization temperature was set at 50°C. Pretreatment with lysozyme (20,000 U/mg; Amersham Life Science) and mutanolysin (5,000 U/ml; Fluka, St. Gallen, Switzerland) was not adopted, as tests with the study samples showed that these protocols did not result in an increase in fluorescence intensity. Hybridization stringency conditions were improved empirically by increasing the formamide concentration in the hybridization buffer (23). Optimal stringencies to enhance the specificity of the designed probe were adjusted by hybridization against cells of reference strains using different formamide concentrations. *Burkholderia vietnamiensis* ATCC BAA248 and *Burkholderia cepacia* ATCC 10856 were chosen as reference strains, as they belonged to the group of closest relatives of PG-01, whose 16S rRNA sequence had the smallest number of mismatches with probe Pand822 (Table 1).

Probe-conferred signal intensities of the cells were normalized by cell area. Mean values of specific signal intensities between different strains were further normalized by the signal of the probe EUB338 hybridized at a 0% formamide concentration to correct for differences in ribosomal content (11, 29). Sufficient stringency for discrimination against nontarget sequences was considered achieved when the fluorescence intensity of nontarget cells was in the background range.

In addition, a negative control (lacking a probe) was prepared to monitor autofluorescence. For combinations of probes with different optimal hybridization stringencies, two hybridizations were performed successively. The first hybridization was performed with the probe which required the higher formamide concentration and higher hybridization temperature, and this was followed by a second hybridization at a lower stringency.

Confocal laser scanning microscopy (CLSM). Images of the granules were acquired with a Fluoview300 confocal laser scanning microscope (Olympus). The fluorescence of the FITC-labeled EUB338 probe was detected by excitation with an argon laser at 488 nm and a long-pass filter (530 nm). The fluorescence of the Cy5-labeled Pand822 probe was detected by excitation with a helium-neon laser at 632.8 nm and a long-pass filter (660 nm). A helium-neon green laser at 543 nm with a long-pass filter (560 nm) was used to detect the TRITC-labeled ARCH519 probe.

Total and probe-conferred cell counts. Granule samples were converted as efficiently as possible to a suspension of individual cells, or at least to smaller cell clusters (6, 7), by suspending 200 mg (wet weight) of granules in a 2-ml microcentrifuge tube topped with Milli-Q water and then shaking the granules for 10 min with a Mini BeadBeater (Biospec Products) at high speed followed by vortexing thoroughly for 10 min in a 15-ml tube. The sample was then diluted 10 times with Milli-Q water, and a 5-μl aliquot was directly immobilized on a gelatin-coated microscopic slide for hybridization with Cy5-labeled probe Pand822 and then with probe EUB338. The samples were then observed with CLSM. Cell counts were determined by evaluating at least 20 microscopic fields with at least 100 cells per field for both EUB338-labeled and Pand822-labeled cells. The ratios of Pand822-labeled cells to EUB338-labeled cells were then calculated.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper have been deposited in the National Center for Biotechnology Information GenBank database under accession numbers AY566574 to AY566587.

RESULTS

Isolation of bacteria from aerobic granules. Based on colony morphology, color, and size, all colonies from the phenol-digesting aerobic granules obtained by a direct isolation technique on MP medium (supplemented with 500 mg of phenol $liter^{-1}$) agar plates were screened into 18 distinct colonies. The colonies were then subjected to partial 16S rRNA gene sequencing and rep-PCR analysis to identify identical strains, and 10 distinct rep-PCR patterns were obtained. A final set of 10 strains, designated PG-01 to PG-10, was chosen for further study. Each strain in the final set had a unique rep-PCR signature. Epifluorescence microscopy and scanning electron microscopy show that three strains (PG-03, PG-06, and PG-10) were bacterial rods, while the other seven strains were cocci (Table 2).

Only colonies associated with strain PG-01 appeared on plates with dilution factors higher than 10^6, indicating that PG-01 had the highest number of active culturable cells in aerobic granules compared to the other nine isolates. Strain PG-01 was estimated to be present in the phenol-degrading aerobic granules at a cell density of $5.64 \times 10^{10} \pm 0.87 \times 10^{10}$ cells g of granules of VSS $^{-1}$. This density is equivalent to 0.056 ± 0.009 g (dry weight) of cells g of granules of VSS^{-1} or $5.6\% \pm 0.9\%$ of granules (g dry weight/g VSS), based on calculated relationships between total cell numbers and the OD_{600} and between biomass concentration and the OD_{600} for a batch culture of strain PG-01 grown on phenol. The fact that strain PG-01 represented 5.6% of granules (on a dry weight basis) suggested that it constituted a significant fraction of the total bacteria residing in the granules.

Physiological analyses of isolates from aerobic granules. Specific growth rates and phenol degradation rates were compared for the 10 isolates at a phenol concentration of 250 mg $liter^{-1}$, which was the concentration in the reactor at the start of each SBR cycle. Table 3 shows that PG-01 had the highest specific growth and phenol degradation rates of 0.185 h^{-1} and 0.292 g of phenol g (dry weight)$^{-1}$ h^{-1}, respectively. The specific growth rates of PG-02, PG-03, PG-05, and PG-09 ranged between 0.06 and 0.11 h^{-1}. The rest of the isolates had specific growth rates less than 0.05 h^{-1}.

Since aerobic granulation represents a form of cell self-

TABLE 2. Taxonomic and phylogenetic characterization of isolates

Isolate	No. of the highest dilution plate of colony appearance	Minimum cell density (CFU g of VSS^{-1})	Morphology	Gram stain[a]	Closest relative	Taxonomic affiliation(s)	16S rRNA gene sequence identity (%)	No. of bases used to establish identity
PG-01	10^7	$(5.64 \pm 0.87) \times 10^{10}$	Cocci	−	*Pandoraea apista* strain LMG 16407	β-*Proteobacteria*	98.7	1,326
PG-02	10^5	$(1.01 \pm 0.92) \times 10^8$	Cocci	+	*Propioniferax innocua* ATCC 49929	Actinobacteria, HGC[b] gram-positive bacteria, *Propionibacteriacaea*	93.5	1,315
PG-03	10^4	$(5.49 \pm 1.80) \times 10^6$	Rod	+	*Rhodococcus erythropolis* strain HV1 00/50/6670	Actinobacteria, HGC gram-positive bacteria, *Nocardioidaceae*	99.8	1,433
PG-04	10^4	$(3.05 \pm 1.42) \times 10^6$	Cocci	+	*Propionibacterium cyclohexanicum* strain IAM 14535	Actinobacteria, HGC gram-positive bacteria, *Propionibacteriacaea*	87.7	1,370
PG-05	10^5	$(1.53 \pm 1.37) \times 10^7$	Cocci	−	*Xenophilus azovorans* KF46FT	β-*Proteobacteria*	98.8	1,437
PG-06	10^5	$(2.55 \pm 1.32) \times 10^6$	Rod	−	*Acidovorax avenae* ATCC 29625	β-*Proteobacteria*	97.9	1,437
PG-07	10^4	$(1.93 \pm 0.72) \times 10^5$	Cocci	−	*Xanthomonas axonopodis* strain s53	γ-*Proteobacteria*	98.1	1,409
PG-08	10^5	$(3.56 \pm 1.52) \times 10^6$	Cocci	−	*Comamonas* sp. D22	β-*Proteobacteria*	97.0	1,408
PG-09	10^4	$(7.62 \pm 2.80) \times 10^6$	Cocci	−	*Pigmentiphaga*	β-*Proteobacteria*	99.6	1,432
PG-10	10^4	$(4.56 \pm 1.72) \times 10^6$	Rod	−	*Hydrogenophaga palleronii* DSM 63	β-*Proteobacteria*	98.5	1,483

[a] Gram negative, −; gram positive, +.
[b] HGC, high G+C content.

immobilization, the autoaggregation abilities of the 10 isolates were also examined. Generally, strains with autoaggregation ability can contribute to the structural stability of cell aggregates. Table 3 shows that most of the isolates were nonflocculated strains. Strains PG-02, PG-04, and PG-06 exhibited autoaggregation activity as floc-like biomass was visualized. Of all the isolates, PG-08 possessed the strongest autoaggregation ability. Autoaggregation took place when PG-08 was cultivated in a shaking incubator in phenol medium or YEPG medium and cell aggregates with a compact structure and a mean size of 0.45 mm were formed. Both polysaccharides contents and polysaccharide-to-protein ratios were high in the ECPs of autoaggregates and were 152.3 mg g (dry weight)$^{-1}$ and 7.5, respectively, in phenol medium and 137.6 mg g (dry weight)$^{-1}$ and 6.7, respectively, in YEPG medium. Cell aggregates possessed good settleability, as these aggregates settled immediately to the bottom of the reagent bottle after shaking was ceased for only 1 min. The OD_{600} of the supernatant was about 0.05, indicating that most cells were associated with the aggregates.

Phylogenetic analysis of isolates. Nearly complete 16S rRNA gene sequences were obtained for the 10 isolates. The sequences ranged from 1,315 to 1,483 nucleotides in length. The putative division and nearest relatives of the 10 sequences were investigated by BLAST, and the results are summarized in Table 2. The isolates fell into three major lineages of the *Bacteria* domain: the β- and γ-*Proteobacteria* and gram-positive high-G+C bacteria. The majority of isolates (6 of 10) were placed in the β subclass of *Proteobacteria*, one isolate belonged to the γ subclass of *Proteobacteria*, and three were gram-positive high-G+C bacteria.

Detection of dominant species by DGGE. The bacterial populations were detected by isolation of DNA from the phenol-acclimated activated sludge seed, from the mature granules, and from isolated strains and then by performing a DGGE analysis of amplified 16S rRNA gene fragments. No PCR amplicons were detected with *Archaea*-specific primers PRA46f and PREA1100r (32). Figure 1 shows the DGGE profiles of the activated sludge, granules, and isolates. DNA extraction and DGGE experiments for community DNA were performed in replicate, and identical fingerprint patterns were obtained for the activated sludge and granule samples. Approximately 10 dominant populations were present in both activated sludge

TABLE 3. Specific growth and phenol degradation rates and autoaggregation activities of isolates

Isolate	Specific growth rate[a] (h^{-1})	Specific phenol degradation rate[a] (g of phenol g [dry weight]$^{-1}$ h^{-1})	Autoaggregation activity[b]	
			Growth in phenol	Growth in YEPG medium
PG-01	0.185 ± 0.003	0.292 ± 0.011	−	−
PG-02	0.095 ± 0.010	0.158 ± 0.015	+	+
PG-03	0.113 ± 0.004	0.146 ± 0.007	−	−
PG-04	0.036 ± 0.003	0.049 ± 0.005	+	−
PG-05	0.065 ± 0.006	0.089 ± 0.010	−	−
PG-06	0.011 ± 0.002	0.014 ± 0.006	+	+
PG-07	NG[c]	ND[d]	ND[d]	+
PG-08	0.009 ± 0.004	0.012 ± 0.005	++	++
PG-09	0.065 ± 0.003	0.083 ± 0.008	−	−
PG-10	0.046 ± 0.005	0.059 ± 0.007	−	+

[a] Specific growth rates were calculated for an initial phenol concentration of 250 mg liter^{-1}.
[b] No flocculation activity, −; flocculation activity, +; strong flocculation activity, ++.
[c] NG, no growth.
[d] ND, no data.

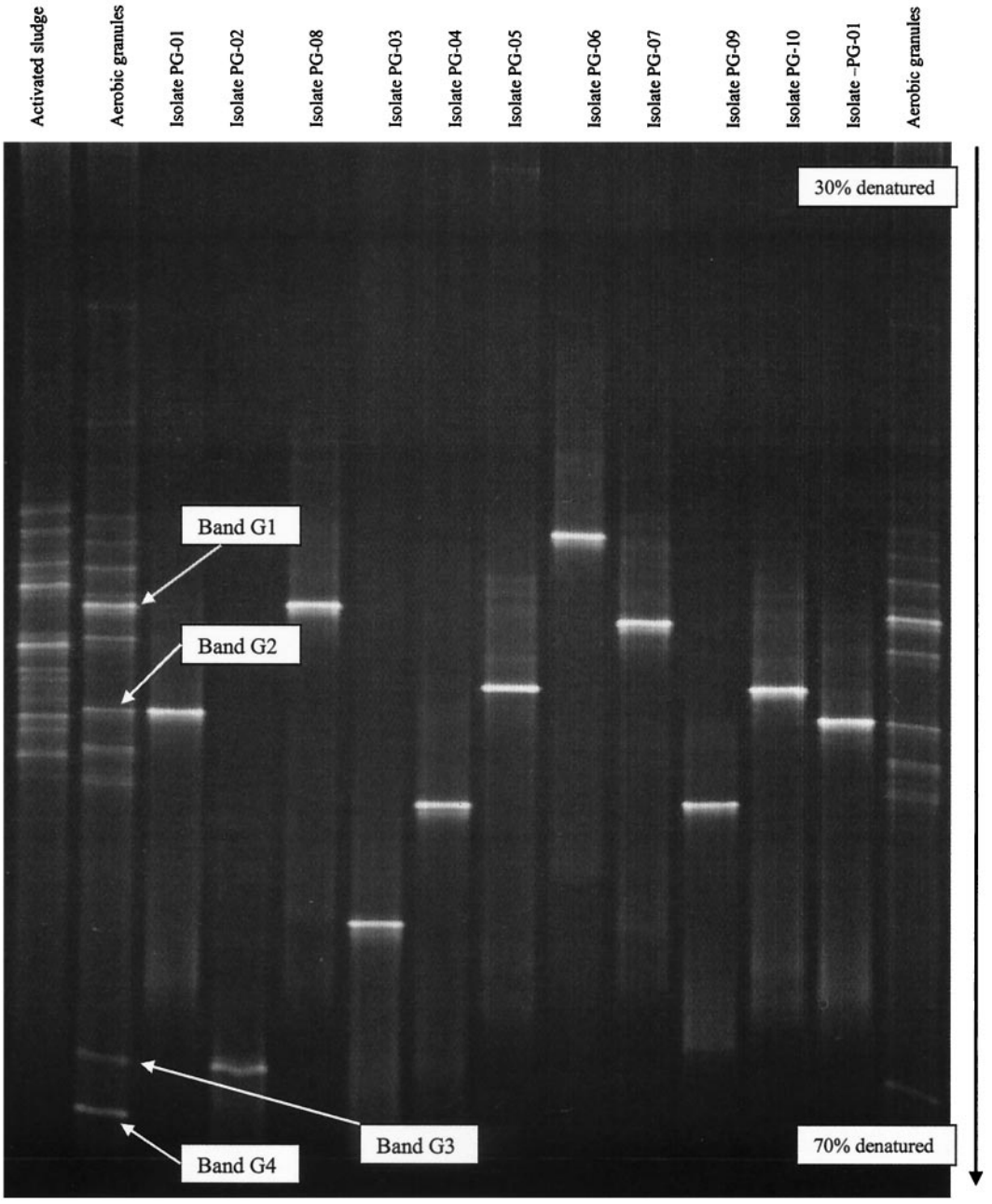

FIG. 1. An ethidium bromide-stained 10% polyacrylamide denaturing gradient gel (30 to 70%) with DGGE profiles of 16S rRNA gene fragments after PCR amplification of nucleic acids from acclimated activated sludge, from aerobic granules, and from individual isolates. The source of each fingerprint is indicated above each lane.

and granules. However, the dominant DGGE bands associated with the activated sludge did not comigrate with the dominant bands from the granules. Moreover, the granules had two new discernible DGGE bands that migrated further into the denaturant gel than other bands. This finding indicated that the major populations in the activated sludge were distinctly different from those in the mature granules. Microbial community adaptation towards the granule form of existence occurred as selection pressures in the SBR forced the looser and less dense seed sludge to aggregate into dense, compact granules. In addition, the DGGE experiments suggested that the seed sludge and the aerobic granules contained microbial communities with a relatively low diversity, as each community was dominated by approximately 10 bands (Fig. 1).

Bands associated with strains PG-08, PG-01, and PG-02 comigrated with bands G1, G2, and G3, respectively, from the aerobic granules. Bands from the other seven strains did not comigrate with DGGE bands from both activated sludge and granules. Bands G1, G2, and G3 (Fig. 1) were isolated and reamplified by PCR for DNA sequencing. Band G4 was also selected for DNA sequencing, as this band appeared at very high denaturant concentrations and might represent a gram-

TABLE 4. Sequence analysis of selected DGGE bands from granules

DGGE band	Length (bases)	Phylogenetically related organism (accession no.)	Sequence identity (%)	Matching isolate
G1	215	*Comamonas* sp. strain 23310 (AJ251577)	97	PG-08
G2	180	*Pandoraea* sp. CCUG (AF268170)	99	PG-01
G3	199	*Propioniferax innocua* (AF227165)	95	PG-02
G4	180	*Salinospora* sp. CNB440 (AY040617)	94	Nil

positive high-G+C species. Before DNA sequencing, the recovered DGGE bands were run on a DGGE gel to confirm their positions relative to the original sample. This step was repeated at least three times to obtain a pure DNA product for sequencing. Partial 16S rRNA gene sequences of approximately 180 to 215 nucleotides were obtained from the four dominant bands. The partial sequences of bands G1, G2, and G3 were similar to the corresponding sequences from isolates PG-08, PG-01, and PG-02, respectively. A comparison with nucleotide sequences from GenBank indicated that G1 was identical to a taxonomically unidentified member of the β subclass of *Proteobacteria* and was 97% identical to *Comamonas* sp. strain 23310 (Table 4). G2 was 99% identical to *Pandoraea* sp. LY, a member of the β subclass of *Proteobacteria*. G3 was 95% identical to *Propioniferax innocua* and belonged to the suborder *Propionibacterineae* of gram-positive high-G+C bacteria. G4 was 94% identical to *Salinospora* sp. CHN964, which was also a gram-positive high-G+C bacterium.

Hybridization optimization with probe Pand822. The effect of increased formamide concentrations on the fluorescence intensity conferred by Cy5-labeled probe Pand822 hybridized with target strain PG-01 and the two nontarget reference strains is shown in Fig. 2. Probe binding to the target strain decreased as formamide concentrations exceeded 20%, attaining background intensities at about 40% formamide. Probe-conferred fluorescence of the reference strains was in the background range at 15% formamide. Nontarget strains can thus be discriminated at 15% formamide. However, since there were no intensity losses, a concentration of 20% formamide was used in subsequent hybridization experiments.

Distribution and function of PG-01 within aerobic granules. FISH-CLSM was used to elucidate the abundance and spatial distribution of strain PG-01 in the aerobic granules. In situ hybridization was performed with thin 20-μm sections by using the FITC-labeled EUB338 probe, the TRITC-labeled ARCH915 probe, and the Cy5-labeled Pand822 probe. The granules consisted of a dense layer of cells surrounding a less dense central region. This structural pattern was repeatedly observed in all sections analyzed. FISH quantification showed that these granules consisted entirely of bacterial cells. Bacteria that hybridized with the EUB338 probe were distributed across the entire granule section, but archaeal signals were not detected with the ARCH915 probe, even in the granule core.

Probe Pand822 hybridized positively to a number of cells, with the same morphology as the PG-01 cocci, in the granule sections examined. Most PG-01 cells were distributed in clusters in the outer layers of the granules (Fig. 3), although several isolated cells of PG-01 could also be detected in the granule interior. Direct counting of probe-hybridized cells after disruption of granules revealed that PG-01 cells were numerically abundant in the granules, accounting for 4.1% ± 3.2% of all EUB338-hybridized cells.

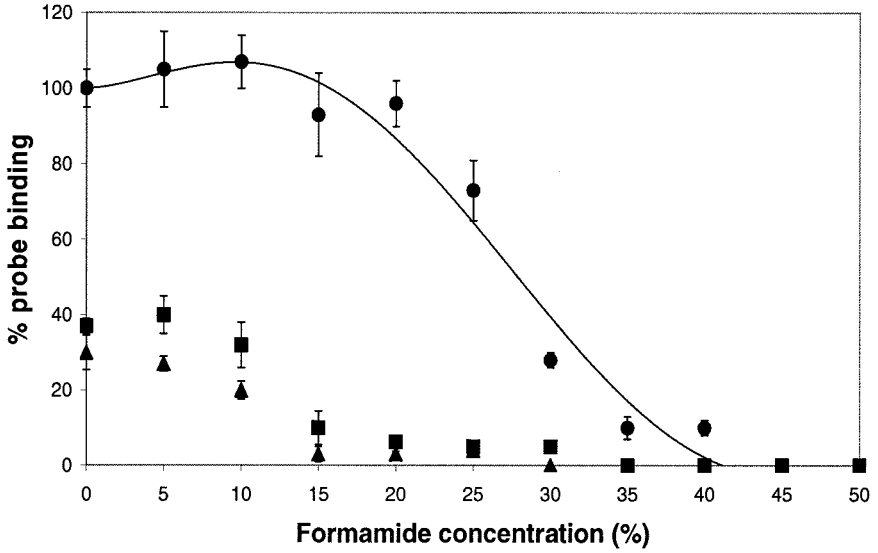

FIG. 2. Signal quantification of Cy5-labeled Pand822 probe to reference organisms. Fixed cells were hybridized with increasing concentrations of formamide at 5% increments. Fluorescence intensities were normalized relative to the signal strength of EUB338 at 0% formamide. The brightness of isolate PG-01 exhibiting maximal probe binding is set as 100%. (●) isolate PG-01; (■) *B. vietnamiensis* ATCC BAA248; (▲) *B. cepacia* ATCC 10856.

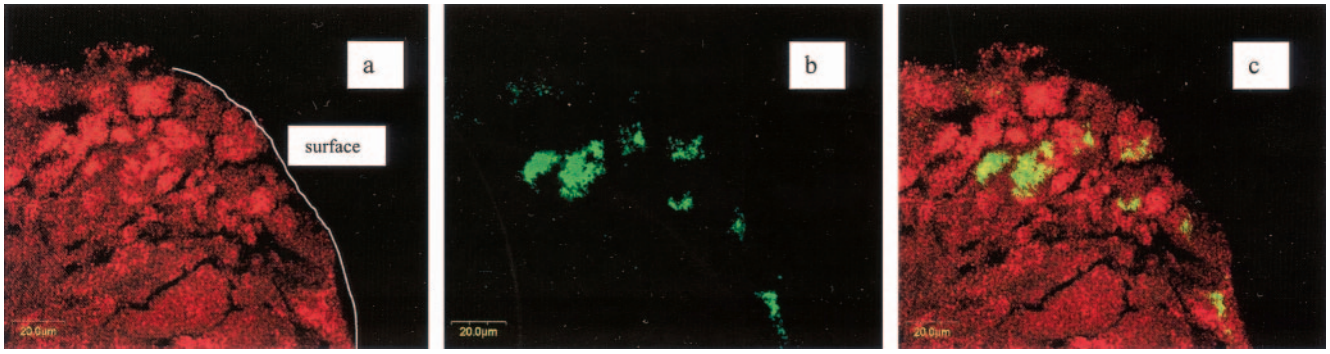

FIG. 3. FISH-CLSM image of outer section of granule (magnification, ×800). The section was simultaneously hybridized with probe EUB338 and probe Pand822. (a) The red area represents cells hybridized with probe EUB338; (b) the green area represents cells hybridized with probe Pand822; (c) a combination of the two images.

DISCUSSION

Bacterial diversity of phenol-degrading granules. A final collection of 10 distinct phenol-degrading isolates cultivated from the aerobic granules was assembled after screening with rep-PCR. Seven of the 10 isolates belonged to the β- or γ-*Proteobacteria* group. These culture-based data are in agreement with previous studies which used FISH or cloning strategies to show that β- and γ-*Proteobacteria* constitute a large fraction of the bacteria in wastewater treatment plants (3, 36) or in glucose-fed aerobic granules (39). Members of β-*Proteobacteria* have also been implicated in phenol degradation in activated sludge, as demonstrated in isolation experiments (43).

One striking observation was the prevalence of gram-positive high-G+C bacteria, which constituted 3 of the 10 isolates cultivated from the phenol-degrading aerobic granules. Based on their strong representation among the granule-based isolates, the gram-positive high-G+C bacteria appeared to occupy a common niche within the aerobic granules. In contrast, gram-positive high-G+C bacteria were not dominant members in phenol-degrading activated sludge systems (43, 44, 46). These observations could probably be explained by the fact that high-G+C bacteria preferred to grow in attached biofilms rather than remain in a planktonic state (20, 41). These microorganisms are also known to be resilient to external stresses because of the presence of a strong cell envelope (47). In addition, several gram-positive high-G+C strains are known to consume soluble chemical oxygen demand rapidly and store it as storage polymers to survive low-nutrient conditions (21, 26). These competitive traits can allow the gram-positive high-G+C bacteria to thrive in situations with low food-to-microorganism ratios, such as in this study, where phenol was completely consumed within the first 30 min of each 4-h cycle (14).

Dominant populations of β-*Proteobacteria* and the gram-positive high-G+C group within the granule community were also confirmed by partial sequencing of selected DGGE bands from the aerobic granules, as shown in Table 4. In addition, the microbial community residing in the aerobic granules was also notable for the absence of cells belonging to the *Archaea* domain. These microorganisms were not detected by PCR or FISH-CLSM techniques, suggesting that an ecological niche could not be established to support the survival and growth of *Archaea* microorganisms.

Functional model of phenol-degrading aerobic granules. Results of direct isolation, DGGE analysis of community DNA, and FISH-CLSM with a probe specific for PG-01 provided independent evidence to support the contention that PG-01 was a numerically important microorganism in the aerobic granules. The high specific growth rate and high specific phenol degradation rate of PG-01 were probable reasons for its numerical abundance in the granules. In view of its high abundance and high catabolic activity, PG-01 could be regarded as a functionally dominant strain and might have contributed significantly to phenol degradation in the granules. Bioaugmentation of PG-01 into aerobic granules improved the phenol degradation ability by 30 to 50% (data not shown). The important function of this strain is intrinsically linked to its distribution within the granules. Phenol concentrations were higher near the granule surface than in the granule interior due to the presence of a substrate concentration gradient (13). Bacteria are known to distribute themselves in mixed biofilms according to who can survive best in the particular microenvironment (45). Therefore, bacteria with high phenol biodegradation ability and high growth rates may be arranged, like strain PG-01, near the granule surface as part of the overall community's scheme to counter toxicity due to high phenol concentrations. Concurrently, slow-growing species or competitively inferior populations can take refuge and accumulate inside the aerobic granules, similar to what happens in biofilms (25, 30).

Efforts to identify functionally important populations in microbial communities have largely focused on aspects relating to the catabolism of toxic substrate (16, 40) and nutrient removal (6, 15), although several studies have been published that took a different route and analyzed the microbial populations that functioned to support the structural integrity of biofilms (35) and activated sludge flocs (33). In the case of aerobic granules, a compact and stable granular structure is known to provide good biomass retention and protection against phenol toxicity (13) and should also be important for the satisfactory performance of aerobic granule-based treatment systems. Although strain PG-08 had the lowest recorded specific growth rate and phenol degradation rate among all the isolates, it was singled out as having the strongest autoaggregation behavior. This finding suggested that PG-08 may be functionally important in maintaining the structural integrity of phenol-degrading aero-

bic granules, particularly since cells with good autoaggregation ability have also been shown to possess good adhesion ability (34). The dominance of PG-08 was also supported by the finding that its 16S rRNA gene sequence matched that of a dominant band from a DGGE fingerprint of the aerobic granules. Moreover, PG-08 has a high extrapolysaccharide content, which can contribute to the formation and stability of aerobic granules (38). Autoaggregation has been suggested to be a microbial response to phenol toxicity in the case of *Pseudomonas putida* CP1 (10). However, PG-08 grows well and forms autoaggregates easily with or without phenol present, indicating that its autoaggregation behavior is regulated in a different way. The precise functional nature, in situ distribution, activity, and metabolism of this strain within the granule community will have to be resolved in a future study.

By examining both phenol degradation and autoaggregation ability in the 10 strains, a trade-off in the two functions became apparent. None of the isolates had both a high growth rate or high phenol degradation rate and high autoaggregation activity. Strains with a high growth rate or a high phenol degradation rate generally had little or no autoaggregation activity. Conversely, strains with a poor growth rate or a poor phenol degradation rate generally had good or strong autoaggregation activity. For example, strains PG-04 and PG-10 showed moderate phenol degradation rates and possessed moderate autoaggregation activity when cultivated on either phenol or YEPG medium only. In the extreme, strain PG-01 had the highest phenol degradation rate and no autoaggregation activity, while strain PG-08 had the lowest phenol degradation rate and exceptionally high autoaggregation activity. Similar trade-offs in species performance of different ecological functions are known to allow different species with different functions to coexist in microbial communities (17). These trade-offs exist because the benefits of performing one ecological function well (e.g., phenol degradation) come at the cost of performing another function (e.g., autoaggregation ability).

Taken together, the above findings lead to the postulation of a simple functional model of the microbial community within the aerobic granules. Phenol degradation and structure stabilization are two basic functions in the aerobic granules. The functional microorganisms within the aerobic granules generally fall into two groups. One group of microorganisms is mainly responsible for phenol degradation while the other group is mainly responsible for maintaining granule structure. At the same time, there may be some functional overlaps as some microorganisms may demonstrate modest levels of both functions. All these microbial groups cooperate with each other to ensure the formation and stability of aerobic granules exposed to high phenol concentrations in the bulk milieu. Within the granules, the bacteria will distribute and organize themselves to best meet the needs of each other and the community (45). Fast-growing bacteria with a good appetite for phenol will distribute themselves near the granule surface, where phenol concentrations are higher. In fact, the structural adaptations and interrelationships that exist in highly structured biofilms have caused them to be regarded as multicellular organisms with developed internal interdependencies and coordinated activities (37).

This kind of model has important implications for system management and can provide a basis for a more knowledge-driven approach to improve the design of granule-based systems for practical application. For instance, high shear force is an environmental selection pressure that is known to be essential for granule formation (38). However, the application of a high shear force is generally associated with a high energy requirement. If conditions can be created to stimulate the growth of those microorganisms responsible for the production of polysaccharides and cell aggregation, aerobic granules can conceivably be formed more economically in the presence of weaker environmental selection pressures.

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